Characterization of low viscosity polymer solutions for microchip electrophoresis of non-denatured proteins on plastic chips

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In this paper, we study characteristics of polymers (methylcellulose, hypromellose ((hydroxypropyl)methyl cellulose), poly(vinylpyrrolidone), and poly(vinyl alcohol)) with different chemical structures for microchip electrophoresis of non-denatured protein samples in a plastic microchip made of poly(methyl methacrylate) (PMMA). Coating efficiency of these polymers for controlling protein adsorption onto the channel surface of the plastic microchip, wettability of the PMMA surface, and electroosmotic flow in the PMMA microchannels in the presence of these polymers were compared. Also relative electrophoretic mobility of protein samples in solutions of these polymers was studied. We showed that when using low polymer concentrations (lower than the polymer entanglement point) where the sieving effect is substantially negligible, the interaction of the samples with the polymer affected the electrophoretic mobility of the samples. This effect can be used for achieving better resolution in microchip electrophoresis of protein samples. © *2011 American Institute of Physics*. [doi:10.1063/1.3668233]

INTRODUCTION

Polymeric substrates are preferred for fabrication of microchips for electrophoresis of biological samples due to the ease with which polymeric microchips can be mass-produced and their relatively low production costs.[1–4] However, microchip electrophoresis of proteins in polymeric chips presents a serious problem when using untreated microchips due to protein adsorption onto the wall of the microchannels. Irreversible adsorption of proteins on the microchannel surface deteriorates the separation performance in uncoated polymeric microchips. Also sample adsorption onto the polymeric microchip surface causes a non-uniform ζ–potential over the length of the channel which can cause band broadening by local variation of the electroosmotic flow (EOF).[5,6] To avoid these negative effects, the surface in the most commonly used polymeric microchip must be modified for suppression of this highly undesirable wall interaction of charged and also uncharged molecules.[7–19]

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There are two common methods to modify the microchip wall: static coating and dynamic coating. Dynamic coating of polymeric microchips using solutions of water soluble polymers is attractive due to its simplicity and the possibility that the same properties of the wall surface can be gotten for each new run by introducing a new solution of the polymer into the microchannels. Hydrophobic polymers such as methylcellulose (MC) which do not adsorb strongly onto normal capillary surfaces are very well adapted to coat hydrophobic surfaces of polymeric microchips.[10–12] More hydrophobic polymers such as poly(vinylpyrrolidone) (PVP) or poly(N,N-dimethylacrylamide) (PDMA) form more stable adsorbed coatings, but are more likely to interact with hydrophobic patches on the surface of folded proteins which reduces separation efficiency.[13]

For this report, we chose microchips made of poly(methyl methacrylate) (PMMA) which is one of the most common polymeric substrates for fabrication of microchips and it has been applied for protein separation.[6,14–16] Previously our group reported a covalent coating on PMMA microchannels by polyethyleneglycol (PEG) which successfully controlled protein adsorption on surfaces,[6] and dynamic coating by using linear polysaccharides such as cellulose derivatives which have a self-coating ability has been applied to prevent sample adsorption on the surface of PMMA microchips.[17–19] As well, we systematically characterized spontaneous adsorption of surfactants and cellulose on PMMA surface using atomic force microscopy (AFM) and infrared external-reflection (IR-ER) spectroscopy.[20]

Despite substantial research effort,[21] the understanding of nonspecific adsorption of proteins onto microchannel surfaces remains incomplete. Nonspecific interactions between surfaces and proteins usually involve hydrophobic interactions,[22] although electrostatic interactions may be present as well with charged or polar surfaces, or in the presence of an electric potential applied to the surface.[23]

We have been studying different aspects of electrophoretic separation of non-denatured protein samples on plastic microchips using high and low viscosity polymers.[24] In the current research, we chose a series of water soluble polymers with different hydrophilicity for application to microchip electrophoresis of protein samples in a PMMA microchip. We studied different characteristics of the microchannels such as protein adsorption, wettability, EOF, and separation efficiency for separation of protein samples in solutions of these polymers.

EXPERIMENTAL SECTION

Reagents and preparation of polymer solutions

Trypsin inhibitor (TI, MW = 20.1 kDa, pI = 4.6), bovine serum albumin (BSA, MW = 66.3 kDa, pI = 4.7), human serum albumin (HSA, MW = 66.3 kDa, pI = 4.7), polysorbate 20 (Tween-20), Tris-borate EDTA (TBE) buffer 10×, MC with viscosity of ~4000 cP, hydroxypropyl methylcellulose (HPMC) with viscosity of ~4000 cP, poly(vinyl alcohol) (PVA) with molecular weights ranging from 30 000 to 70 000 g/mol, and PVP with molecular weights of 360 000 g/mol were all purchased from Sigma Chemical Co. (St. Louis, MO). They differed in chemical structure (Fig. 1). The polymer solutions were prepared according to the method suggested by the manufacturer. Dilution of the TBE stock concentrate to a 1 × TBE running buffer resulted in a buffer containing 89 mM Tris-borate and 2 mM EDTA, pH 8.3. The viscosity of each polymer solution was summarized in Table I. To reduce sample adsorption, we also prepared MC with an additional 0.01% Tween-20 (MC-T).

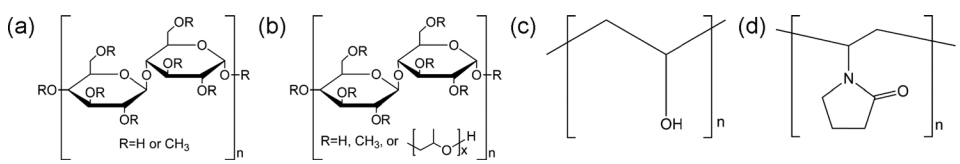

FIG. 1. Structure of each polymers; (a) MC, (b) HPMC, (c) PVA, and (d) PVP.

TABLE I. Comparison of viscosity in different polymer solutions. 0.2% was used for EOF measurements and 0.2%-1.0% was used for separation experiments.

		Concentrations (%)				
	Type of polymer solutions	0	0.2	0.4	0.8	1.0
Viscosity (cP)	MC	0.8[a]	2.4	5.0	13.1	32.5
	HPMC		2.1	3.7	9.2	29.5
	PVP		1.1	1.4	1.9	2.3
	PVA		0.9	1.0	1.3	1.5

[a]0% concentration was the viscosity of 1 × TBE.

Protein labeling

The protein samples were labeled with Alexa Fluor 647 using a protein labeling kit (Invitrogen, Tokyo, Japan). The kit consisted of an amine-reactive dye, sodium hydrogen carbonate, a purification resin, and an elution buffer. However, we have not use the purification resin and the excess dye remained in the sample. The concentration of labeled protein and extent of labeling were measured using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Following the labeling the protein/dye conjugate were stored at 4 °C until applied to microchip electrophoresis.

Microchip electrophoresis

A plastic microchip made of PMMA (i-chip 3 DNA, Hitachi Chemical Co. Ltd., Tokyo, Japan) was used, and its PMMA substrate was commercially available (Aclara, Mountain View, CA).[25] The channel cross section was 100 μm (width) by 30 μm (depth), and effective separation length was set at 30 mm. The distances from the intersection of the microchannels to the buffer reservoir, buffer waste reservoir, sample reservoir, and sample waste reservoir were 5.7, 37.5, 5.2, and 5.2 mm, respectively. The microchip electrophoresis was done on the Hitachi microchip electrophoresis system SV 1210 (Hitachi Electronics Engineering Co. Ltd., Tokyo Japan) equipped with laser induced fluorescence (LIF) detection (excitation and emission wavelength is 650 and 680 nm, respectively).

Contact angle measurements

The contact angle of water was measured with a CA-D type face-contact angle meter (Kyowa Interface Science Co. Ltd., Saitama Japan). The PMMA surface was first treated with 10 μl of dynamic coating, incubated for 5 min, and then washed with a sufficient amount of dd-H2O to remove un-bonded polymers and ions from the PMMA surface. After the surface was completely dried at room temperature, 2 μl of ultrapure water was placed on the PMMA surface using a sampler. The contact angles were measured immediately after placement on the polymer surface. Each value reported was the average of a minimum five measurements.

Protein molecules adsorption test

In order to measure the amount of protein molecules adsorbed to the microchip wall, we developed a method based on desorption of adsorbed protein molecules via washing the chip wall surface with sodium dodecyl sulfate (SDS) micelles and measuring the fluorescence. For the experiments given here, a final pH of 8 was chosen for the background electrolyte. At this pH, HSA is well above pI, so it has a substantial negative charge. A 200 μg/ml solution of HSA conjugated with Alexa Fluor 647 was made up in 20 mM Tris-HCl including 0.025% of the dynamic coating polymer. The solution was placed in the microchannel well for 5 min and then washed three times with 10 μl of the background buffer in order to eliminate all unbounded protein molecules. Then the microchannel well was washed 5 times with 10 μl of

10% SDS in 20 mM Tris HCl buffer (pH = 8.3). The fluorescence was measured with a FP-6500 fluorescent spectrofluorometer (JASCO, Japan). The experiment was repeated for dynamic coatings using MC, PVP, PVA, and HPMC.

EOF measurements

Current monitoring was used to measure EOF in the PMMA microchannels.[26] In a typical measurement, the microchannels and all reservoirs except the sample waste reservoir were filled with the same volume of a buffer (e.g., 1 × TBE). The sample waste reservoir was filled with a five-fold dilution buffer (e.g., 0.2 × TBE). A HVS448 1500V power supply (LabSmith, Livermore, CA) was employed to provide high voltage during the measurement, and current variation was recorded every 20 μs using a personal computer and Sequence software program (LabSmith). The time required for current to reach a plateau state was used to calculate the linear velocity (cm s^{-1}). The EOF mobility (cm^2 V^{-1} s^{-1}) was calculated by dividing this linear velocity by the electric field strength (432.69 V cm^{-1}).

RESULTS AND DISCUSSION

Quantitation of nonspecific protein adsorption onto the PMMA surface

To evaluate coating efficiency of the dynamic coatings, we compared the amount of fluorescent protein which adsorbed onto uncoated and coated microchannels. We measured the fluorescent intensity of the eluted protein samples from the uncoated microchannels and postulated this fluorescence as complete or 100% adsorption. Table II shows the amount of protein adsorbed onto the microchannels in the presence of each polymer solution in comparison with the uncoated microchannels. We used 0.025% solution of each polymer as dynamic coating since by increasing the concentration of some of these polymers the protein adsorption was negligible and it was not possible to differentiate coating efficiencies of different polymer solutions. For example we could see that MC-T efficiently suppressed the protein adsorption, and the suppression efficiency was around 95% compared to the bare PMMA microchannels. The next-best polymer that suppressed protein adsorption was HPMC and the protein adsorption was less than 10%. MC coating suppressed protein adsorption to less than 20% of the amount of protein. These results showed us that derivatives of cellulose had a good suppression efficiency and compared to the uncoated microchannels, they provided good adsorption suppression. The difference between MC and HPMC is the presence of another substituent group, polypropyleneglycol (PPG) (–$OCHCH_3CH_2$) and that between MC and MC-T is surfactant, Tween-20. The features of PPG are similar to those of PEG, which is frequently used to prevent the protein adsorption, and therefore HPMC should better suppress protein adsorption compared to MC. Likewise, surfactant is used to prevent the protein adsorption, and therefore MC-T should better suppress protein adsorption compared to MC.

To clarify the effect of polymer structure on coating, we used simple derivatives of vinyl polymer; these were PVA and PVP, having a hydroxyl and a pyrrolidone group, respectively.

TABLE II. Comparison of amount of protein adsorbed onto the PMMA channel surface, water contact angle on the PMMA surface, and electroosmotic flow in the PMMA microchannels when using different dynamic coatings (n = 5).

	Protein adsorption (%)	Water contact angle (deg)	EOF mobility × 10^{-5} (cm^2 V^{-1} s^{-1})
Uncoated PMMA	100[a] ± 1.0	70.3 ± 0.5	9.51 ± 0.48
MC-T	5.2 ± 0.1	50.3 ± 2.8	0.74 ± 0.07
MC	16.1 ± 0.2	58.1 ± 1.0	0.73 ± 0.07
HPMC	9.4 ± 0.1	59.0 ± 2.0	0.87 ± 0.09
PVP	51.3 ± 0.5	64.5 ± 1.4	1.04 ± 0.17
PVA	9.9 ± 0.1	54.7 ± 1.6	8.21 ± 0.30

[a]The amount of protein adsorbed onto the uncoated microchannels was considered as 100% adsorption.

The biggest difference in their characteristics is hydrophilicity (a detailed discussion is given in the following section). PVA has a stronger hydrophilic feature than PVP, and showed protein adsorption of 10%, the same as HPMC. In contrast to PVA, PVP showed protein adsorption of 50%. These results suggested that PVP was less efficient for preventing protein adsorption onto the PMMA microchannels than PVA. Generally, the protein easily adsorbed onto the hydrophobic PMMA microchannels due to its hydrophobic interaction, and therefore PVA could suppress the protein adsorption much more than PVP.

Water contact angle on the surface of coated PMMA

Also in Table II, the measurement results of water contact angle on the surface of PMMA after coating with different polymers are compared. All the dynamic coatings increased hydrophilicity of the PMMA surface. In particular, a rather hydrophilic surface was achieved using HPMC, MC-T, and PVA solutions as dynamic coating and the surface in the presence of PVP was still hydrophobic. Taking the features of substituents and side chains in polymers into consideration, we could relate the contact angle results to the protein adsorption results discussed earlier. In the case of adding surfactant, for MC-T the hydrophobic group would be oriented in the same direction as the PMMA surface and the hydrophilic group would be toward the opposite side (water side). Such a condition prevented the protein adsorption and had a contact angle of 50°. PVA is hydrophilic because it contains a hydroxyl group, and it permitted protein adsorption to less than 10% for the water contact angle around 54°. Although the difference of the contact angle between HPMC and MC had no statistical significance, HPMC showed less protein adsorption than MC. The reason why less protein adsorption did not lead to a low contact angle was attributed to the PPG group in HPMC. Since the PPG group could prevent protein adsorption efficiently but would be more hydrophobic than hydrogen (–H) or methyl (–CH3), groups, HPMC could suppress the protein adsorption and MC had a low water contact angle. The pyrrolidone group in PVP is more hydrophobic than any other side chain or substituent in the polymers of our experiments. The water contact angle of PVP was 64° and it prevented only 50% of the protein adsorption due to its hydrophobicity. These results supported our presumption that an efficient coating should increase the hydrophilicity of the PMMA surface. Comparing the water contact angle measurements and protein adsorption test results showed that higher hydrophilicity did not always mean higher efficiency for preventing protein adsorption due to the features of substituents or side chains in the polymers. However, we could estimate the amount of protein adsorption from the tendency between the protein adsorption and hydrophilicity.

EOF in coated PMMA microchannels

High EOF in microchip electrophoresis can be a disadvantage for negatively charged proteins because it acts as a counter flow which means longer separation times and broadening of the sample zone due to the diffusion. In the worst case, where the counter flow is higher than the electrophoretic mobility the analyte may not migrate toward the detector. And moreover, the right value of EOF is mandatory to have resolution of very similar proteins, as it is the case of isoforms of glycoproteins.[27]

Therefore we measured the EOF in the PMMA microchip in the presence of different polymer solutions. The EOF is surface-driven flow, and therefore the direction of EOF indicates the charge feature and the value of EOF shows the charge distribution near the PMMA surface. The EOF mobility for the uncoated PMMA microchannels was $(9.51 \pm 0.48) \times 10^{-5}$ cm^2 V^{-1} s^{-1} (RSD for 5 measurements was 5.02%) and the direction of the EOF was from the anode to the cathode (opposite that of the electrophoretic mobility of protein samples). The results (Table II) showed that using 0.2% PVA solution in the microchip, we obtained the EOF which was almost the same value as for the uncoated microchip $(8.21 \pm 0.30) \times 10^{-5}$ cm2 V^{-1} s^{-1}. The PVA coating could not change the charge distribution on the PMMA surface. This result showed us that the hydroxyl group of PVA should be oriented to the solvent direction in this experimental condition and partially dissociated due to its pKa value (pKa; = 10.67).[28] But the

EOF in the presence of 0.2 % PVP or 0.2% HPMC or 0.2% MC decreased by nearly one order of magnitude. Coating with PVP, HPMC, and MC could efficiently change the distribution of negative charge near the PMMA surface. The less hydrophilic side chain group in PVP and the sterically bulky cellulose structure in HPMC and MC might contribute to the EOF decrease. For MC-T, we showed that Tween-20 at the concentration used here (0.01%) did not affect the EOF. Also we saw that although the PVA solution effectively suppressed protein adsorption and increased hydrophilicity of the PMMA surface, looking at the EOF results we could conclude that the high EOF for the PVA solution might cause longer separation time and possible band broadening.

In summary, our results from the three basic tests for evaluation of dynamic coatings showed that the values suggested that HPMC and MC-T were the best dynamic coatings in terms of suppressing protein adsorption onto the PMMA surface and getting low values of EOF. However, one last and important question still remains and that is how these polymers might affect the separation of protein samples in the PMMA microchip if they are used as dynamic coating and at the same time, at higher concentrations as separation media.

Microchip electrophoresis of protein samples

Figure 2 shows two examples of microchip electrophoresis of a non-denatured protein marker BSA and TI conjugated with Alexa Fluor 647 in different polymer solutions of 0.2%. Compared with the non-coating microchannel (data not shown), separation in all coated microchannels was successfully achieved; there were 3 peaks in the microchip electrophoresis of BSA conjugated with Alexa Fluor 647 and 5 peaks in TI conjugated with Alexa Fluor 647. BSA conjugated with Alexa Fluor 647 appeared as two peaks, and that could be because of labeling. We have many lysines on BSA and it is possible to have different version of labeleled BSA. It would be much more than only two peaks, and then the wide peak includes many close version of BSA. TI conjugated with Alexa Fluor 647 also appeared as multiple peaks due to the labeling process. TI has multiple reaction sites on it for the fluorescent dye, and therefore different ratios of fluorescent dye to TI make multiple peaks.

The electropherograms in this figure showed that a better coating efficiency was achieved using HPMC and MC-T solution that showed lower protein adsorption, higher hydrophilicity, and lower EOF in the series of polymer coatings we studied. As shown in Tables III and IV, the migration time of peak 3 and 4′ in PVA solution was much longer, and the full width at half maximum (FWHM) in PVA was much wider than that of other polymer solutions. The

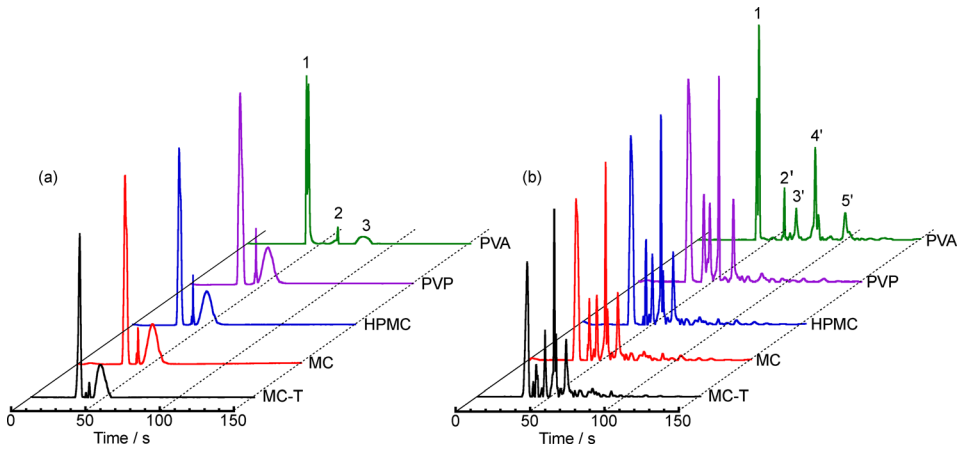

FIG. 2. Microchip electrophoresis of (a) BSA conjugated with Alexa Fluor 647 and (b) TI conjugated with Alexa Fluor 647 was done under the non-denaturing condition in different polymer solutions of 0.2% (peak 1 free is for fluorescent dye and the 2nd set of peaks (2-3 and 2′-5′) is due to different modifications of BSA and TI). Electrophoresis was done in TBE buffer. Injection and separation were done by applying fields of 440 V/cm and 335 V/cm, respectively.

TABLE III. Migration time, and the ratio of FWHM of polymer solutions to PVA solution, theoretical plate number, and resolution in the electropherograms of BSA conjugated with Alexa Fluor 647 in different dynamic coatings (Fig. 2(a)).

	Migration time of peak 3 (s)	Ratio of FWHM of polymer solutions to PVA solution at peak 3	Theoretical plate number of peak 3	Resolution between peaks 1 and 3
PVA	77.63	1	468.24	4.44
PVP	51.83	0.868	276.76	2.19
HPMC	50.13	0.816	293.35	2.31
MC	49.81	0.855	263.53	2.15
MC-T	47.13	0.789	276.85	2.22

reason why the separation of only PVA showed a longer migration and peak broadening was that the higher EOF in this case, which is a counter flow to the electrophoretic mobility, increased the separation time and hence, broadening of the peaks.

With the objective of resolution, a better resolution was achieved using PVA solution which has the highest EOF in the series of polymer coatings we studied. In this concentration of polymer solution (0.2%), polymers which we used in this time, PVA, PVP, HPMC, and MC could not entangle with each other.[18,29–32] Below entanglement point of polymer solution, the interaction frequency between samples and polymer solutions is important to get a better resolution. In the separation of PVA, the higher EOF which is a counter flow to the electrophoretic mobility increased the separation time and hence, increased the interaction frequency and achieved a better separation of the peaks. In the electrophoresis below entanglement threshold of polymer solutions, counter flow can play different roles: it can increase the separation time, which is equivalent to doing a separation in a longer channel, and it can get a better resolution for the samples. However, much higher EOF can also cause too long a separation time and therefore peak broadening and in the worst case the sample will not enter the separation channel (the effect which we saw in uncoated PMMA chip where high EOF hinders electrophoretic migration of the samples).

To the best of our knowledge, there has been few reports on the electrophoresis of proteins in polymer solution in concentrations under entanglement point,[33] but there was no report about the microchip electrophoresis of non-denatured protein in such conditions. In Fig. 3, we compared relative mobility of two protein samples BSA (66 kDa) and TI (20 kDa) and a small fluorescent dye (Alexa Fluor 647) in different concentrations of polymers indicated in the figure. We saw that when using MC as the separation media and when the concentration of MC was higher than its entanglement point (0.2%),[18] there was a decrease in mobility; however, until 1% the sieving effect was not yet considerable. We also showed that MC and MC-T in term of suppressing EOF and protein retardation were similar.[18] For the other three polymers, we did not expect to have any sieving effect because their concentration was much lower than their

TABLE IV. Migration time, and the ratio of FWHM of polymer solutions to PVA solution, theoretical plate number, and resolution in the electropherograms of TI conjugated with Alexa Fluor 647 in different dynamic coatings (Fig. 2(b)).

	Migration time of peak 4′ (s)	Ratio of FWHM of polymer solutions to PVA solution at peak 4′	Theoretical plate number of peak 4′	Resolution between peaks 1 and 4′
PVA	78.68	1	13896	17.74
PVP	53.37	0.591	18300	6.20
HPMC	52.40	0.500	24641	6.58
MC	51.75	0.545	20196	6.44
MC-T	51.19	0.455	28452	6.86

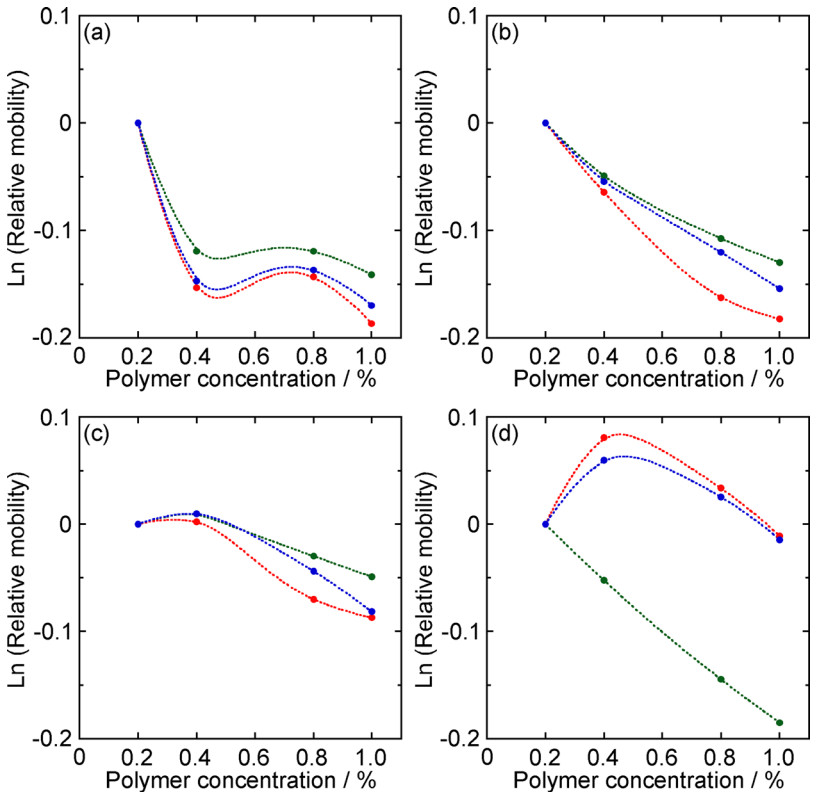

FIG. 3. Relative mobility (mobility in a given concentration of polymer/mobility in 0.2% of polymer) of two protein samples (BSA and TI) and fluorescent dye (Alexa Fluor 647) in different concentrations of polymer solution; (a) MC, (b) HPMC, (c) PVA, and (d) PVP. Green, red, and blue showed dye, BSA, and TI, respectively. Electrophoresis was done in TBE buffer. Injection and separation were done by applying fields of 440 V/cm and 335 V/cm, respectively.

reported entanglement points; entanglement threshold of PVA, PVP, and HPMC are more than 1%.[29–32] With HPMC, which has a rather amphiphilic characteristic with both hydrophilic and hydrophobic groups, we observed that by increasing the concentration the relative mobility of the three samples decreased because the interaction between HPMC and the proteins or fluorescent dyes increased due to the increase of both hydrophilic and hydrophobic groups along with the increase of the concentration of HPMC. However, for PVA which is very hydrophilic, retardation of the three samples did not change by changing the polymer concentration because the highest EOF in the series of polymer coatings could make enough interactions to the three samples in these concentrations ranging from 0.2 to 1.0%. For PVP which is hydrophobic we saw a linear decrease of mobility of the fluorescent dye, but not for the proteins. This could be explained by comparison of the hydrophobic properties of the fluorescent dye to the proteins and the higher possibility of interaction of the dye with the hydrophobic groups in PVP.

Although HPMC and MC-T were the best dynamic coatings from the standpoint of protein adsorption, water contact angle, and low values of EOF, PVA showed the best resolution in these dynamic coatings. Because the used protein samples had large electrophoretic mobility, relatively high EOF (counter flow) resulted in higher resolution. In these concentration ranges from 0.2 to 1.0%, we would not estimate the significant retardation of the samples due to the subthreshold of entanglement points or the unfavorable sieving effect, and therefore the relatively high EOF could be the cause of higher resolution and therefore from resolution point of view PVA was for a better dynamic coating. But, when if higher concentrations of polymers are used, the samples may be retarded by the sieving effect of entangled polymers, and the mobility of samples should also decrease. In such a situation, the effect of low EOF on resolution is much important, consequently HPMC or MC-T will be preferred as they are more efficient for controlling protein adsorption and low EOF.

CONCLUSIONS

Our results here can offer further understanding of the interaction of protein samples toward a PMMA surface and should be useful for choosing an appropriate dynamic coating for separation of proteins using this type of polymeric chip. The results showed that the polymers which have both hydrophobic and hydrophilic parts in their structures were more efficient for suppression of protein adsorption in PMMA microchips. Polymers with more hydrophilic groups such as PVA which may adsorb onto the PMMA surface through their hydrophobic part had less effect for decreasing EOF but were efficient for suppressing the protein adsorption. We also showed that interaction of the polymer with the sample could affect the electrophoretic mobility even though the polymer concentrations was lower than its entanglement point; this finding can be used to develop new polymeric media for achieving better resolution of protein samples in short channels in microchip electrophoresis.

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